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MORPHOLOGIC AND MOLECULAR CHARACTERIZATION OF THE SARCOCYSTS OF *SARCOCYSTIS RILEYI* (APICOMPLEXA: SARCOCYSTIDAE) FROM THE MALLARD DUCK (*ANAS PLATYRHYNCHOS*)

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ABSTRACT: Macroscopic sarcocysts are often observed in ducks, but at present their taxonomic status remains uncertain because ducks serve as intermediate hosts for several such parasites in the genus *Sarcocystis*. One such species, *Sarcocystis rileyi*, was long ago established to involve the northern shoveler duck (*Anas clypeata*) and the striped skunk (*Mephitis mephitis*) as its intermediate and definitive hosts, respectively. Here, we employed light microscopy, electron microscopy, and DNA sequencing to more precisely describe diagnostic attributes of parasites presumed to represent *S. rileyi* occurring in a naturally-infected mallard duck (*Anas platyrhynchos*). By light and transmission electron microscopy, sarcocysts from the mallard duck resembled the *S. rileyi* described from *A. clypeata*. We document 18S, ITS-1, and 28S rDNA sequences from the mallard duck, the first for *S. rileyi* from any host. Sequences of conserved and variable portions of nuclear ribosomal DNA indicated that *S. rileyi* is related to, but distinct from, parasites employing opossums as their definitive host (including *Sarcocystis neurona* and *Sarcocystis falcatula*). Diagnostic ultrastructural features and nucleotide sequences should aid in future studies and communications regarding this parasitic taxon, which lends itself to experimentation because its sarcocysts are macroscopic and easily excised from infected birds.

Infections by species of *Sarcocystis* are considered common in many species of birds (Dubey et al., 1989). Macroscopic sarcocysts in ducks are of particular interest because hunters often bring pieces of infected muscles for laboratory identification; these specimens have been used for teaching since they are macroscopic and easily excised from host tissues. Nonetheless, there remains considerable confusion regarding the identity of such parasites in birds. Macroscopic sarcocysts have been reported from many species of waterfowl from North America, and all of these have been considered to exemplify *Sarcocystis rileyi* (Stiles, 1893) Minchin 1913 (reviewed by Dubey et al., 2003). However, ducks are also known to be intermediate hosts for several other species of *Sarcocystis* (Drouin and Mahrt, 1979, 1980). At present, it is uncertain whether macroscopic sarcocysts in many species of ducks belong to 1, or more, species of *Sarcocystis*.

Life cycles are known for 2 avian species of *Sarcocystis* whose sarcocysts are macroscopic, i.e., *S. rileyi* and *Sarcocystis falcatula*. The type intermediate host of *S. rileyi* is the northern shoveler duck (*Anas clypeata*), and the striped skunk (*Mephitis mephitis*) is its sole definitive host (Cawthorn et al., 1981). The definitive host for *S. falcatula* is the opossum (*Didelphis virginiana*), but the intermediate hosts have been considered to include several avian species, including ducks (Box and Smith, 1982). However, *S. falcatula* is now considered more than 1 species (Dubey and Lindsay, 1999; Dubey, Garner et al., 2001; Dubey, Johnson et al., 2001; Dubey, Rosenthal, and Speer, 2001; Dubey et al., 2003; Mansfield et al., 2008). The species of *Sarcocystis* transmitted via opossum are evolutionarily related, but genetic attributes of *S. rileyi* remain unknown. In order to better understand the correspondence of macroscopic sarcocysts in mallards to that previously reported for *S. rileyi* in northern shovelers, and in order to better describe this taxon and facilitate future research and communication concerning this widely prevalent taxon, we here provide the first report of detailed morphological and genetic characteristics of *S. rileyi* from naturally-infected mallard ducks (*Anas platyrhynchos*).

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MATERIALS AND METHODS

Naturally-infected ducks

Breasts from 2 mallards (*A. platyrhynchos*) shot by hunters on 1 November 2008 were used in the present study. The ducks were more than 1-yr-old and had been shot at the Steamboat Lake State Park, Routt County, Colorado. The breasts were refrigerated over the weekend and shipped cold by overnight mail to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland. The specimens were received at APDL 3 days after death. Both ducks had grossly visible, rice grain-like sarcocysts (Fig. 1A).

Light microscopic examination

For light microcopy, sections of muscles were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and examined after staining with hematoxylin and eosin (H & E).

Transmission electron microscopic (TEM) examination

For TEM, samples of muscle containing macroscopic cysts from both ducks were fixed in Karnovsky's fixative. Tissues were post-fixed in 2% (w/v) osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. Three sarcocysts from duck no. 1 were examined with an electron microscope.

Molecular characterization

Individual cysts from each of 2 ducks were excised and subjected to DNA extraction using DNeasy columns (Qiagen, Inc., Valencia, California) according to manufacturer recommendations. A preliminary analysis of partial small subunit (18S) sequences was performed for each of 10 sarcocysts by directly sequencing PCR amplification products on an Applied Biosystems 3100 Automated Sequencer. Subsequently, full-length sequences of this conserved portion of nuclear rDNA, the large subunit (28S) rDNA, as well as the more rapidly evolving first internal transcribed spacer (ITS-1) were obtained for 1 sarcocyst from each of the 2 mallard ducks. The 18S gene was amplified in 2 segments using primers 18S1F and 18S 11R (Rosenthal et al., 2008) and primers 18S9F-GTTGGTTTCTAG-GACTGA and 18S14-3R-TCACCGGAACACTCAATCGGTAGG. The 28S rDNA was amplified using methods described by Mugridge et al. (1999). To amplify the ITS-1 sequence, primer 18S14F (Rosenthal et al., 2008) was paired with 2 reverse primers: 5.8s-1R-GATGATTCACT-GAATTCTGCAATTCACATT and 5.8s-2R-TTCGCTGCGTTCTTCA TCGATGCGAGAGCCAAGA. After an initial 3 min denaturation at

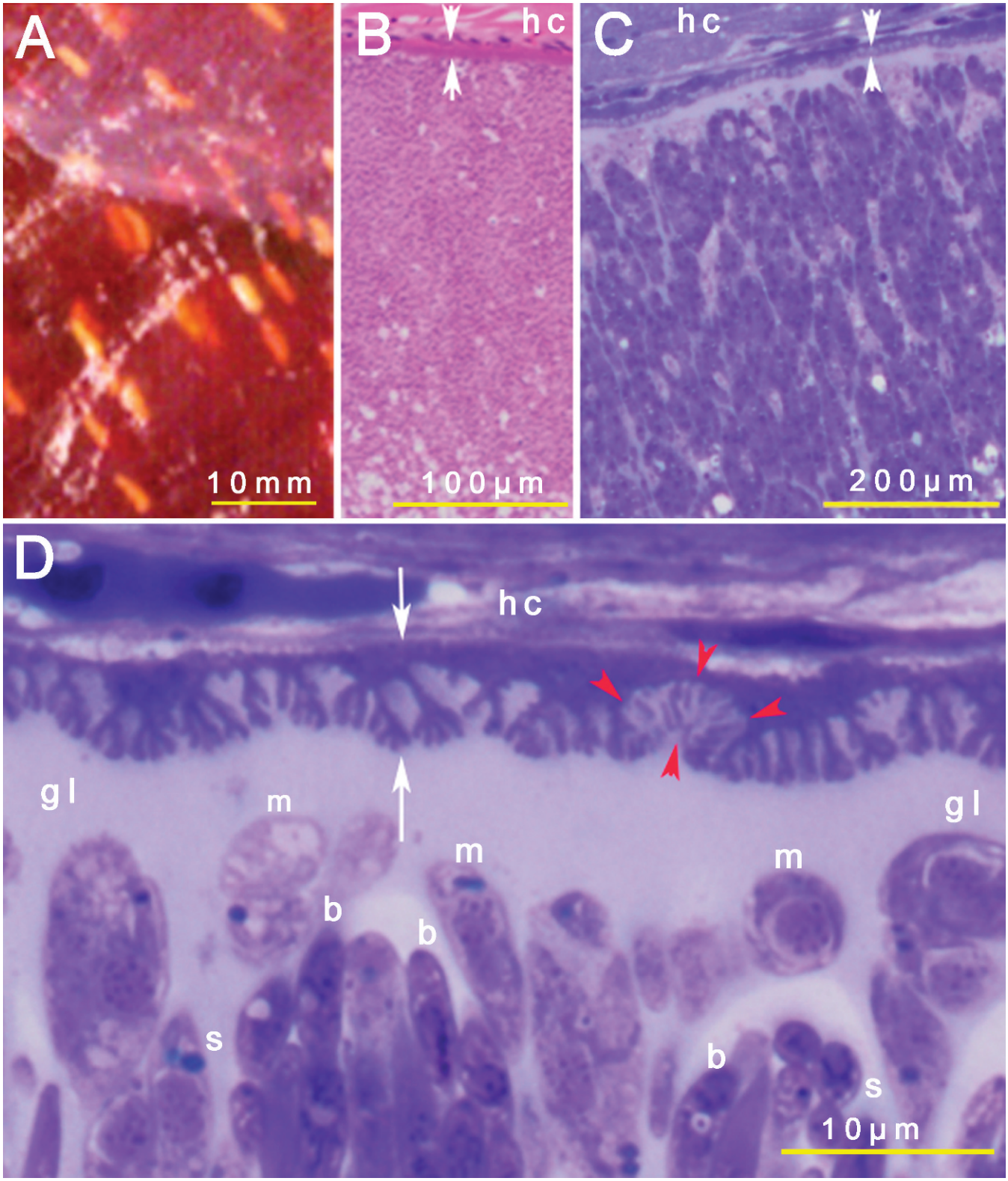

FIGURE 1. Structure of sarcocysts of *Sarcocystis rileyi* from a naturally-infected *Anas platyrhynchos*. (**A**) Grossly visible sarcocysts in pectoral muscle, unstained. (**B, C**) Low power view. The host cell (hc) is closely applied to the cyst wall. The thickness of the sarcocyst wall is indicated by opposing arrowheads. (B) Hematoxylin and eosin stain. (**D**) Higher magnification of the cyst wall in 1-µm section stained with Toluidine blue. Note branching of the villar protrusions on the cyst wall, 1 has the mushroom shape (red arrowheads). Just underneath the villar protrusion is the granular layer (gl) that continues into the interior of the cyst as septa (se). Note faintly stained metrocytes (m) and darkly stained bradyzoites (b).

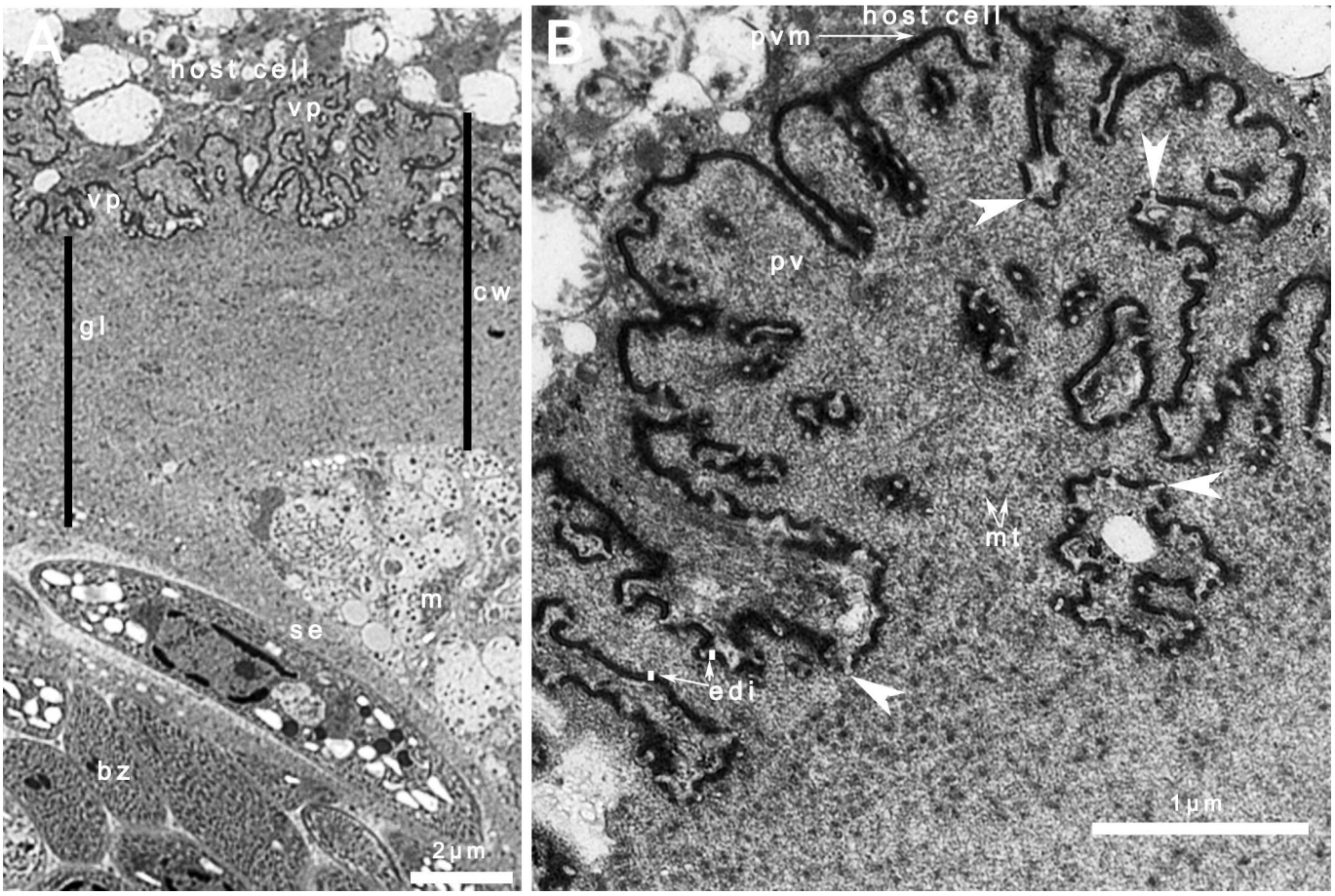

FIGURE 2. Transmission electron micrograph of a sarcocyst of *S. rileyi* in the pectoralis major of a mallard. (**A**) The sarcocyst wall (cw) is thick and has the branched villar projections (vp) and the granular layer (gl). Metrocytes (m) and bradyzoites (bz) are juxtaposed with the granular layer. The host cell is degenerating. (**B**) Higher magnification of the sarcocyst wall showing details of parasitophorous vacuolar membrane (pvm) and villar protrusions (vp). The pvm is lined by electron dense layer (edl) and the lining is interrupted (arrowheads) at irregular intervals. Note fine microtubules or filaments.

94 C, ITS-1 fragments were amplified using 40 cycles of PCR amplification comprised of 94 C for 30 sec, 58 C for 45 sec, and 68 C for 90 sec, followed by a final 10 min extension at 68 C.

Homologous sequences were identified by a BLAST query of GenBank, and a multiple sequence alignment of those sequences most similar to that of *S. rileyi* was constructed using MUSCLE (Edgar, 2004). Phylogenetic relationships were reconstructed from 500 bootstrap replicates under the criterion of minimum evolution and using Kimura 2-parameter distances, as implemented in MEGA4 (Tamura et al., 2007). BLAST was also used to search for sequences similar to the ITS-1 region and to compare such regions obtained from the parasites in each of the 2 ducks.

RESULTS

By light microscopy, the structure of sarcocysts in 5-µm–H & E and 1-µm Toluidine blue-stained sections was similar. The sarcocyst wall was up to 6 µm thick. The morphologic and molecular descriptions are from duck no. 1. In 1-µm Toluidine blue-stained sections, villar protrusions were visible and appeared to be highly branched (Fig. 1C). The granular layer was smooth and homogenous but of uneven thickness (Fig. 1C). Faintly stained metrocytes were located in the granular layer or just below it. The middle zone contained bradyzoites, which were arranged in rows and separated by septa. Bradyzoites were slender; 20 longitudinally cut bradyzoites measured in 1-µm thick sections were 12–14 µm long and approximately 2 µm wide (Fig. 1C). The interior of the sarcocyst contained degenerated zoites.

Ultrastructurally, the outermost layer of the sarcocyst, the parasitophorous vacuolar membrane (pvm), was wavy and lined by 50 nm thick electron dense layer (edl). The edl extended along the length of the sarcocyst wall, although it was absent at invaginations of the pvm (Fig. 2). The sarcocyst wall was highly folded into branched villar projections (vp) (Fig. 2). These vp were up to 4 µm long and coalesced with each other. The vp contained faint filamentous structures and were not apparent in all vp. The basal parts of vp were lined by a granular layer (gl), which was uneven and up to 5 µm thick (Fig. 2A). The gl contained course granules (Fig. 2). A few metrocytes were present at the periphery of sarcocysts.

Numerous bradyzoites were present below metrocytes (Fig. 1C). Single or groups of bradyzoites were separated by septa. The bradyzoites had double membraned pellicle and contained a conoid, micronemes, rhoptries, amylopectin granules, a mitochondrion, dense granules, and a terminal nucleus (Fig. 3). The conoidal and the non-conoidal ends of the bradyzoites were lined by an electron dense layer (Fig. 3). A ring-like structure was seen toward the non-conoidal end of several bradyzoites (Fig. 3).

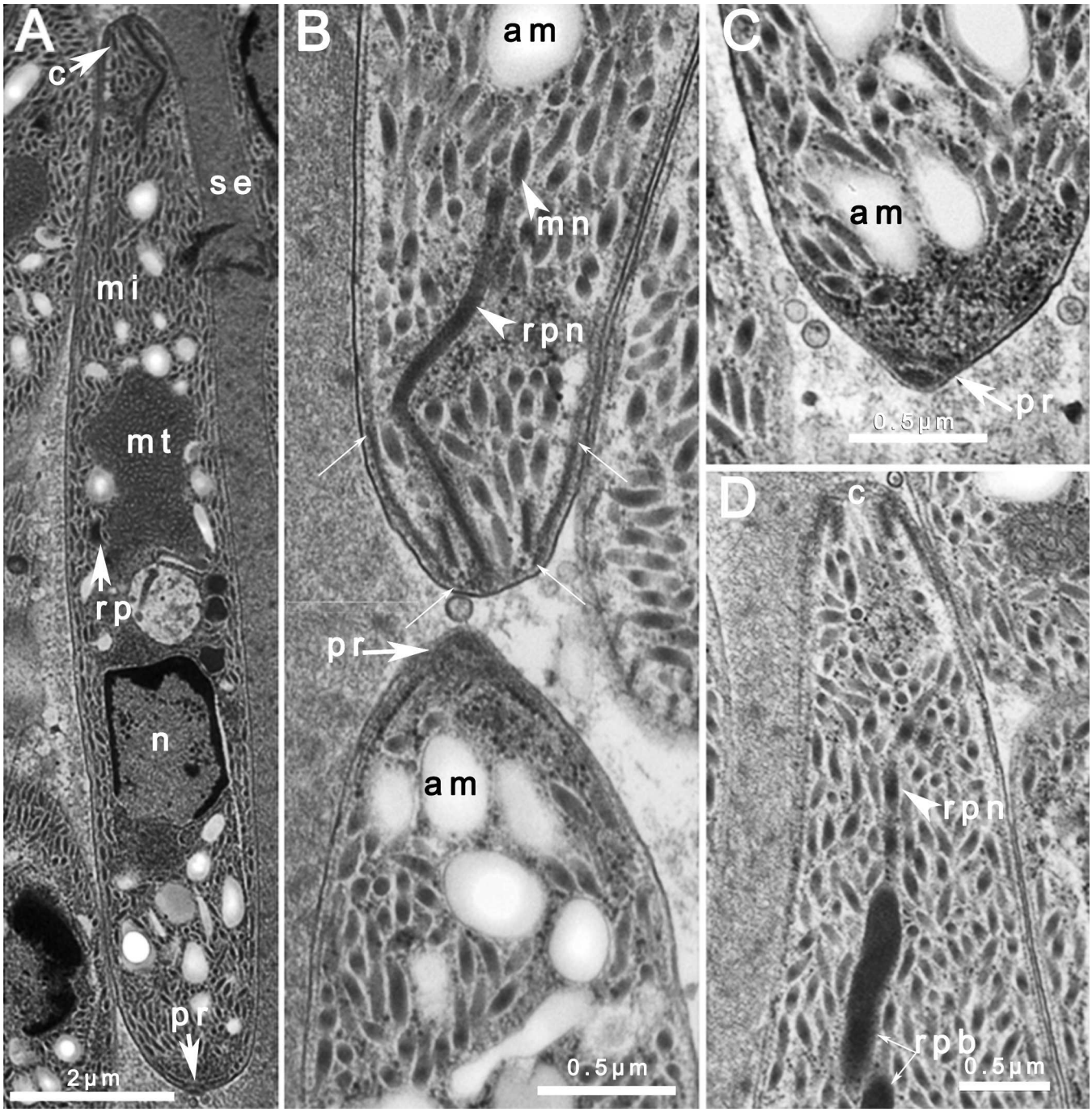

FIGURE 3. Transmission electron micrographs of *S. rileyi* bradyzoites from a duck. Note septum (se) between bradyzoites, conoid (c), thickening of the membrane at both ends of the bradyzoite (slender arrows), a ring-like structure at the posterior end (pr), numerous micronemes (mn), a mitochondrion (mt), rhotries (rp) with a long neck (rhn) opening in the conoid and a bulbous blind end (rpb), amylopectin (am), and a nucleus (n). (**A**) A longitudinally cut bradyzoite. (**B–D**) Higher magnification of conoidal and non-conoidal ends of bradyzoites.

The micronemes were numerous and dispersed throughout the length of the bradyzoite but were most abundant in the 1/3 of the conoidal end of the parasite. The rhoptries had a long neck opening in the conoid and a bulbous blind posterior end (Fig. 3). Only 2 rhoptries were seen in any 1 plane of section.

An initial survey of 18S rDNA from 10 sarcocysts from each of 2 mallard ducks found them to be identical to each other at all 1,195 sequenced base pairs. Subsequent sequencing effort was, therefore, focused on 1 sarcocyst from each duck, resulting in a 1,643 bp fragment that was identical in each (GenBank GU120092). These were very similar (>96%) to homologous sequences derived from various other members of the Sarcocystidae. They were strongly related to other species of *Sarcocystis*; they most closely resembled sequences recently reported from

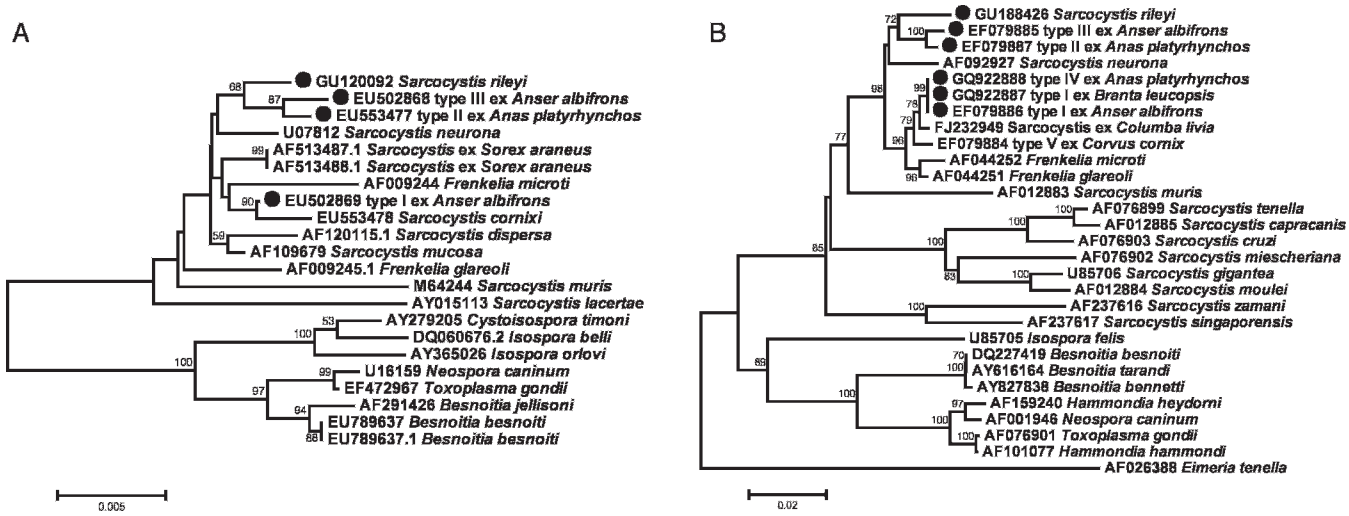

FIGURE 4. Minimum evolution trees reconstructed from variation in (**A**) the 18S and (**B**) the 28S rDNA. BLAST was used to search for homologues of the 18S and 28S rDNA sequences, which were subsequently aligned using MUSCLE and analyzed under the criterion of minimum evolution based on Kimura 2-parameter distances. After complete deletion of gapped positions, a total of 1,549 and 1,148 positions remained in the 18S and 28S datasets, respectively. Taxa derived from anseriforme birds are delineated thus: ●.

parasites in another *A. platyrhynchos* (with cyst type II) and from another anseriforme bird from Europe (*Anser albifrons*) (Fig. 4). Similar results, with greater bootstrap support, were obtained when analyzing variation in the 28S rDNA (GenBank GU188426). In each isolate, the new sequences were related to, but dissimilar from, all previously sequenced isolates; in each case, previous parasite isolates from *A. platyrhynchos* were inferred to be more closely related to other parasites than to the present isolate. The precise historical interrelationships among such closely related species cannot be stated with certainty, as reflected in strong bootstrap support for only certain aspects of the phylogenetic tree.

The ITS-1 sequences derived from each of 2 sarcocysts were identical to each other over 1,077 of 1,079 sequenced bases (GenBank GU188427). A single nucleotide substitution was evident at 1 position; at a second position, more than 1 base difference was evident. Although the first 86 and last 60 bases of this sequence showed close similarity to the end of the 18S and beginning of the 5.8S rDNA subunits, respectively, of various other apicomplexans, the ITS showed little identifiable similarity to other sequences deposited in GenBank.

Histological sections of muscles from duck no. 1 (D5515) and duck no. 2 (D5516) stained with H & E and Toluidine blue were deposited as types in the U.S. National Parasite Collection (USNPC nos. 102433, 102434), U.S. Department of Agriculture, Beltsville, Maryland.

DISCUSSION

As with many early descriptions for coccidian parasites, the original one for *S. rileyi* could not benefit from the precision now achievable through the use of ultrastructural and genetic analysis. Indeed, it may never be possible to know with certainty whether any subsequent analysis is being performed on the taxon originally described. The present effort is further complicated by the fact that the parasites we have examined, using contemporary diagnostic methods, were derived from a duck species only related to the type host for *S. rileyi*. Nonetheless, the close morphological correspondence between the parasites naturally infecting mallard ducks here and parasites employed in successful experimental infections in the type host substantiates the hypothesis that the taxon studied here represents *S. rileyi*.

The striped skunk is the only known definitive host for *S. rileyi*. There is no report of *S. rileyi* infection in ducks from other continents except North America, which is probably related to the geographic range of the definitive host. Kalisińska et al. (2003) reported macroscopic sarcocysts in 1 of 148 ducks in Poland, but these authors did not provide structural details of sarcocysts. Kutkiene et al. (2008) reported macroscopic sarcocysts in a duck from Lithuania, but it was morphologically distinct from *S. rileyi*; sarcocysts were ribbon-like, thin, up to 5 mm long, and ultrastucturally distinct from *S. rileyi*.

Sarcocystis spp. are structurally distinct from all other cyst-forming coccidians because the cyst wall has villar protrusions. Dubey et al. (1989) and Dubey and Odening (2001) classified the villar protrusions into 37 structurally distinct types. The mature sarcocysts of *S. rileyi* have a type-23 tissue cyst wall (Dubey et al., 1989), characterized by anastomizing protrusions that contain fine granules and microfilaments. This type of sarcocyst wall has not been found in any other species of *Sarcocystis* (Dubey et al., 1989; Dubey and Odening, 2001).

Our data undermine the notion that a given intermediate host species is parasitized by only a particular lineage of *Sarcocystis*. Although the material described here maintains clear genetic affinities for other parasites isolated from anseriforme birds, they do not share exclusive common ancestry with those previously characterized from mallard ducks. Instead, the present isolates share unique characters that distinguish them from others available for comparison, which, in turn, manifest their own phylogenetic affinities with parasites of other birds. In this context, it is also interesting to note that *Sarcocystis neurona*, a mammalian parasite that has also been reported in avian intermediates (Mansfield et al., 2008), along with its close relative (*S. falcatula*), occupies a phylogenetic position close to parasites of other birds in their intermediate hosts (*S. rileyi*, and parasites

described from crows, pigeons, geese, and ducks) or definitive host (*Frenkelia* spp.) stages.

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